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Analysis of the *rdd* locus in chicken: a model for human retinitis pigmentosa

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Purpose: To identify the locus responsible for the blind mutation *rdd* (retinal dysplasia and degeneration) in chickens and to further characterise the *rdd* phenotype.
Methods: The eyes of blind and sighted birds were subjected to ophthalmic, morphometric and histopathological examination to confirm and extend published observations. Electroretinography was used to determine age of onset. Birds were crossed to create pedigrees suitable for genetic mapping. DNA samples were obtained and subjected to a linkage search.
Results: Measurement of IOP, axial length, corneal diameter, and eye weight revealed no gross morphological changes in the *rdd* eye. However, on ophthalmic examination, *rdd* homozygotes have a sluggish pupillary response, atrophic pecten, and widespread pigmentary disturbance that becomes more pronounced with age. Older birds also have posterior subcapsular cataracts. At three weeks of age, homozygotes have a flat ERG indicating severe loss of visual function. Pathological examination shows thinning of the RPE, ONL, photoreceptors and INL, and attenuation of the ganglion cell layer. From 77 classified backcross progeny, 39 birds were blind and 38 sighted. The *rdd* mutation was shown to be sex-linked and not autosomal as previously described. Linkage analysis mapped the *rdd* locus to a small region of the chicken Z chromosome with homologies to human chromosomes 5q and 9p.
Conclusions: Ophthalmic, histopathologic, and electrophysiological observations suggest *rdd* is similar to human recessive retinitis pigmentosa. Linkage mapping places *rdd* in a region homologous to human chromosomes 9p and 5q. Candidate disease genes or loci include *PDE6A*, *WGN1*, and *USH2C*. This is the first use of genetic mapping in a chicken model of human disease.

The *rdd* (retinal dysplasia and degeneration) phenotype in chickens was first reported in 1979 in Scottish commercial stocks imported from the USA [1]. Two papers in the early 1980's described *rdd* as a recessive progressive deterioration of the retina [2,3]. Chicks were said to be sighted at hatch but had only limited vision and were noticeably less active than normal birds. Signs of defective vision became more apparent by 8-10 weeks when head nodding and small circling movements developed. Vision deteriorated progressively and by sexual maturity at 15 weeks most birds were blind. In histological sections taken on the eighth day of incubation, holes were observed extending from the retinal pigment epithelium through the inner nuclear layer of the *rdd* retina [3]. By 11 days of incubation, undulations were observed in the outer nuclear, outer plexiform and inner nuclear layers. As incubation progressed the holes grew in number, but they then disappeared within one week of hatching. A marked reduction in photoreceptors compared to normal controls was evident by 18 days of incubation and after hatching (at approximately 21 days incubation) the retina continued to thin due to cell loss from the photoreceptor and inner nuclear layers. In vitro cultures of neural retinal cells from *rdd* homozygous 6-8 day embryos revealed a 30% slower growth rate and increased level of apoptosis compared to cells from normal age-matched controls [4]. These observations led Wilson and colleagues [3] in 1984 to compare *rdd* to human Retinitis Pigmentosa (RP).

Retinitis Pigmentosa is a heterogeneous group of inherited progressive retinal degenerations beginning in the peripheral retina [5]. Patients experience night blindness and loss of visual fields, leading to complete blindness in around 30% of cases and severe visual disability in the remainder. Ophthalmic examination reveals constriction of retinal arterioles, optic disc pallor, and characteristic bone spicule-like pigmentary deposits, while electrodiagnostic testing shows a reduced or abolished rod electroretinogram (ERG) [6]. RP is the most common inherited retinal dystrophy, affecting approximately 1 in

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3500 people or around 2 million sufferers world-wide [7]. Other retinal diseases such as macular and cone-rod dystrophies (CRD) originate in the central retina, causing photophobia and loss of visual acuity [8]. Though these central retinal dystrophies are less common, they share features with Aging Macular Dystrophy (AMD), a common cause of visual disability in the elderly. Leber's Congenital Amaurosis (LCA) is a severe form of retinal dystrophy, causing complete blindness within the first years of life [9]. To date, 132 loci have been implicated in the different forms of human retinal dystrophies, for which 83 of the genes are known (see RetNet). These diseases can be inherited as autosomal dominant, autosomal recessive, X-linked, mitochondrial, and polygenic traits. Together they account for 11.5% of blind registrations under the age of 65 [10].

As well as *rdd*, four other forms of retinal degenerations have been reported in the chicken. These include retinal degeneration in the Rhode Island Red strain *rd* [11], blindness enlarged globe (*beg*) [12], retinopathy globe enlarged (*rge*) [13,14], and the delayed amelanotic strain DAM [15]. One of these loci has been fully characterised genetically and matched with an equivalent human disease. The *rd* phenotype is caused by a null mutation in the photoreceptor guanylate cyclase (*Gucy1*b*) gene and is thus a model for one form of Leber's Congenital Amaurosis type 1 in humans [11]. A second of these strains, *rge*, has been mapped to chicken chromosome 1 by linkage analysis, in a region most probably homologous to human chromosomes 7q31-35, 21q21 or 22q12-21 (Inglehearn et al., unpublished data).

The chicken eye differs from that of a human in a number of ways. Unlike the rod-dominated human retina, avian retinas are generally cone-dominated and are often bifoveate [16]. Approximately 82% of the inner segment area of the chicken retina consists of double-cones, a species of photoreceptor found in all vertebrate groups except placental mammals [17,18]. However the chicken eye is comparable in size to that of a human, which facilitates pathological examination and should simplify the testing of experimental therapies. Furthermore, the availability of many diverse breeds of chickens, each selected for different agricultural purposes, will be of considerable utility when searching for susceptibility loci for human diseases. The level of conservation of gene order between the chicken and human genomes is similar to that between humans and mice, in spite of the much greater evolutionary separation [19]. Therefore the study of spontaneously occurring inherited blindness in chickens as a model for human retinal dystrophies has the potential to make a valuable contribution both to understanding these conditions and to the search for therapies.

The published descriptions of the *rdd* strain are limited to behavioural and histological observations on the trait, and since 1984 no further report exists of this mutant in the literature. We now report the recreation of an *rdd* breeding colony from birds of the original line. With this colony we have confirmed and extended the published descriptions to include ophthalmic, morphological and electroretinographic observations. In addition we have discovered that the inheritance of this trait has been incorrectly assigned and that it is in fact a sex-linked mutation. Finally we carried out linkage analysis to locate the genetic lesion responsible for the *rdd* defect.

METHODS

Animal husbandry: The *rdd* line is maintained at the Roslin Institute as a small pedigree population of 8 single male-female families that are mated in a systematic manner to control inbreeding [20]. The wild-type (sighted) birds were from a line of White Leghorns maintained at the Roslin Institute by random mating of 20-30 individuals per year. Three 3 blind homozygous (*rdd*/*rdd*) males were crossed with 3 female sighted White Leghorns (+/+). One blind (*rdd*/-) female from each family was mated to one of three unrelated White Leghorn males (+/+). The sighted male progeny of these matings (*rdd*/+) were backcrossed to their dams to produce the birds for assessment and genotyping. All matings were by artificial insemination

Classification of blind/sighted birds: Visual function was assessed by three behavioural methods. Firstly, each bird was gently removed from its home cage and allowed to grip the front of the metal food trough in front of the cage. Sighted birds balance easily, turn and re-enter the cage. Blind birds cannot balance easily and do not re-enter the cage but may stumble off the perch. Secondly, the lateral side of the head was moved slowly towards a solid object such as a part of the cage structure. Sighted birds move their necks to keep at a distance from the object whereas blind birds do not take avoiding action. If any doubt remained, the bird was placed on a concrete floor and its activity was monitored. Sighted birds walk purposefully and may try to escape, in marked contrast to blind birds that may not move at all even under gentle pressure. Two people independently performed each assessment of visual function on at least two occasions at 8-10 and 12-14 weeks of age.

Ophthalmic and morphometric analyses: Blind and sighted birds from 3 age groups, of 3-4 months, 6-9 months and 2.5-4 years, were subject to an ophthalmic examination. This was preceded by an initial instillation of the local anaesthetic Amethocaine (1%) in each eye. Intra-ocular pressures were measured with the Tono-Pen XL (TM) tonometer. Axial lengths were measured with the BVI Axis II A-scanner. Corneal diameters were measured, followed by direct and indirect non-mydriatic fundus examination. After death, homozygous and heterozygous birds were subject to an orbital exenteration. The excess adnexal and orbital tissues were cleaned off the globe, and photographs were taken to allow observation of gross morphology. Individual eyes were weighed to evaluate overall volume.

Electroretinography: ERGs were measured in two three-week old, hemizygous females and two three-week old, heterozygous male siblings. Anaesthesia was induced with ketamine hydrochloride (Pharmacia Animal Health, Tadworth, Surrey, UK; 10 mg/kg body weight i/m) and xylazine (Bayer UK Ltd., Newbury, Berkshire, UK; 2 mg/kg body weight i/m). A cotton wick electrode was placed on the front of the cornea after application of physiological saline. Ag/AgCl elec-

trodes were inserted subcutaneously to touch the top of the eye. Single flash stimuli of approximately 10μsec duration were provided by a white light stroboscope (Electronic Applications, London, UK), with the lamp situated 300 mm from the eye. Potentials were recorded on a 5103M oscilloscope (Tektronix Inc., Beaverton, OR) with a DAM 6A preamplifier (WPI Instruments Inc., Stevenage, Hertfordshire, UK) and photographed using a C-5A Oscilloscope camera (Tektronix Inc.).

Pathology: Eyes were fixed in 10% neutral buffered formalin and paraffin embedded by standard techniques. Four micron sections were then stained with haematoxylin and eosin.

Genotyping: Samples of fresh blood were collected at 10-14 weeks of age by superficial venepuncture of a wing vein. DNA extraction was performed using the single-step DNAzol method (Invitrogen, Paisley, UK). Twenty-two microsatellite markers from the Z sex chromosome [21] were typed on individual genomic DNAs from sighted and blind birds. PCR reactions for all microsatellite markers were performed separately in a total reaction volume of 15μl. Reactions contained: 15 ng genomic DNA, 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris.HCl pH 8.3, 0.1% Triton X-100, 0.01% gelatin, 20 mM dNTP, 0.5 Unit of FastStart Taq DNA polymerase (Roche Products Ltd., Welwyn Garden City, Hertfordshire, UK) and 5 pmol of each primer. PCR was performed with a hot start of 15 min at 95 °C, then 35 cycles of 15 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a final step of 30 min at 60 °C. 1μl of each reaction was pooled in 100μl dH_2O with the others in the set. 1μl of this was added to 0.0025μl of the GENESCAN-350 ROX internal size standard and 20μl HiDi formamide (Applied Biosystems, Warrington, Cheshire, UK). Reliable and polymorphic markers were selected and organised into compatible marker sets based on the fragment size and dye colour of the PCR product. The PCR products from the same animal were diluted, pooled and size-fractionated on an ABI 3700 automated sequencer. Fragment sizes were calculated relative to the ROX-350 internal size standard by using GeneScan 3.5.1 DNA fragment analysis and Genotyper 2.5 software. All pedigree, marker genotypes and trait data were recorded in resChick, a generic resource database [22]. This database was used to format data for genetic linkage analyses.

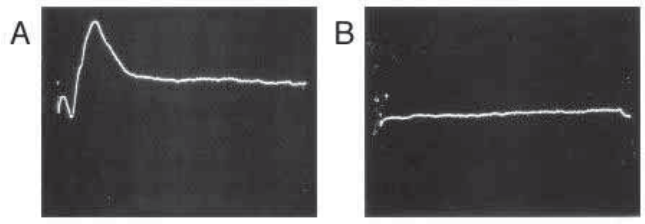

Figure 1. Electroretinographs of *rdd* heterozygous male and hemizygous female birds. Heterozygous 3-week-old male birds (*rdd*/+) have a normal ERG (**A**), whereas their age-matched hemizygous female siblings (*rdd*/-) showed complete absence of an ERG response (**B**).

Linkage analysis: All data logged in the resChick database was imported to Crimap for linkage analysis. This happened in three phases. (1) Two-point analysis was carried out to determine whether there was significant linkage between markers. (2) Build analysis determined the most likely order of the markers and position of the *rdd* region. (3) A fixed analysis was done, where the *rdd* locus was moved along a selected length of the Z chromosome to determine its most likely position.

RESULTS

Segregation ratio: After mating a blind male from each family to a wild-type White leghorn female, male progeny were found to be sighted while females were blind, indicating sex-linked inheritance. This contradicted previous publications in which the *rdd* trait was described as autosomal recessive in inheritance [2,3,23]. However on careful examination, the crosses described by Randall and co-workers [2], while consistent with autosomal recessive inheritance, are in fact equally consistent with Z-linked inheritance. These authors crossed blind hens (*rdd*/-) with sighted (carrier) cocks (*rdd*/+) and obtained a 1:1:1:1 ratio of sex and affectation status, but the reverse cross described above (*rdd*/*rdd* males against +/- females) revealed the linkage between sex and the trait.

For subsequent mapping studies, the heterozygous males from this cross were backcrossed to the blind dams and a total of 77 progeny were classified, of which 39 were blind (18 males and 21 females) and 38 sighted (21 males and 17 females). These figures are consistent with the 1:1 ratio expected under a model of sex-linked recessive Mendelian inheritance.

Ophthalmology: Ophthalmic examinations were carried out on 3 blind birds aged 2 months, 11 aged 6-10 months and 8 aged 2.5 years and over. Both sexes were approximately equally represented in each group except the older birds, which were all male. In addition 11 sighted carriers aged 6-10 months and 8 wild type controls aged 11 months were examined, again with approximately half of each sex in each group. No significant differences were observed between sighted carriers and controls. Homozygous *rdd* birds aged 2 months had pupils that were sluggish or non-reactive to light stimulus, widespread diffuse “pepper-dust” pigmentation and an atrophic pecten. The pecten is a highly vascularised comb-like structure found in all birds, which projects from the optic nerve head into the posterior chamber. It is an organ of nutrition that probably equates to the inner retinal vasculature of the human eye, since the avian retina is avascular. Homozygotes aged 6-10 months once again had sluggish pupils and an atrophic pecten. Pigmentary disturbance was more pronounced, with evidence of chorioretinal atrophy, particularly around the base of the pecten, fundus pallor and small punched-out punctate chorioretinal lesions. Four out of 11 birds of this age also had either posterior subcapsular or anterior cortical lens opacities. *rdd* homozygotes over 2.5 years of age consistently had sluggish or absent pupillary responses, an atrophic pecten and dense posterior sub-capsular cataracts, as a result of which the fundus view was generally poor. Where visible, the retina appeared atrophic, with a greyish hue and pigment spots.

Morphology: Mean measurements for intra-ocular pressure, axial length and corneal diameter were determined for each sex, using blind birds aged 6-10 months (n=11), blind birds aged 3-4 years (n=8, males only) and sighted birds aged 11 months (n=8). There were no differences between the two sexes for any trait. Axial lengths and intra-ocular pressures were similar in blind and sighted birds but corneal diameter was significantly greater in sighted compared with blind birds at less than 1 year. In the older male blind birds however, corneal diameters were greater than those of either blind or sighted younger males. The mean weights of the eyes of blind and sighted male chicks were 3.1 g and 2.8 g respectively at 3-4 months and 2.9 g and 2.6 g at 7-9 months of age (sed 0.055 g, n=4). The effect of age was not significant whereas sight vs. blind approached formal significance (P=0.055). Eye weights as a proportion of body weight in blind birds averaged 1.14, 1.49, 1.50, 1.48 and 1.46 g/kg respectively at hatch, 3-4 months, 7-9 months, 3 years and 4 years of age. Post-hatch relative weights in wild type (White Leghorns) aged 3-4 months were 1.51 (se 0.12) g/kg. Thus the *rdd* mutation, unlike the Retinopathy Globe Enlarged (*rge*) and Blindness Enlarged Globe (*beg*) forms of chicken inherited blindness, is not associated with globe enlargement.

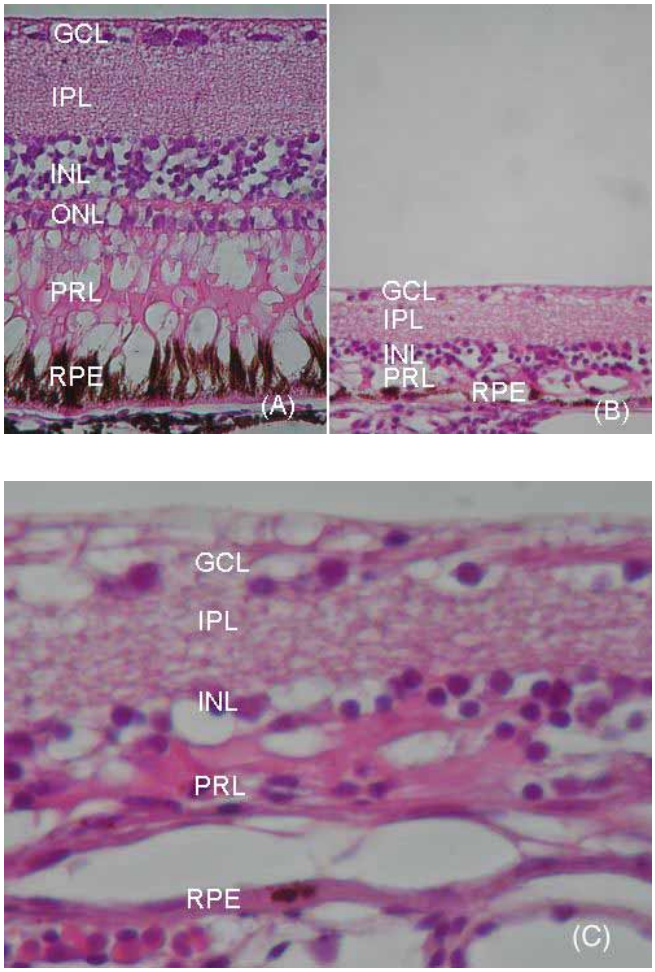

Figure 2. Comparison of the pathology of normal and *rdd* chicken retinas at 3-4 months. Comparison of normal (**A**) and *rdd* (**B**) retina at 3-4 months of age, both to scale. The *rdd* retina shows marked atrophy of all layers with virtually complete loss of photoreceptors and replacement by gliosis. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, PRL = photoreceptor layer and RPE = retinal pigment epithelium. Both haematoxylin and eosin at 340x magnification. (**C**) High power view of the *rdd* retina showing marked atrophy. Note in particular the complete absence of the outer nuclear layer (usually seen between the inner nuclear and photoreceptor layers). The photoreceptor layer shows loss of photoreceptors, gliosis and prominent clefts above the remnant of the retinal pigment epithelium. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, PRL = photoreceptor layer, RPE = retinal pigment epithelium. Haematoxylin and eosin at 1,800x magnification.

Electroretinography: The progeny of a cross between an affected *rdd* male and a normal female were subjected to ERG testing at 3 weeks. Two females showed a complete absence of an ERG response, while two of their male siblings had normal ERGs (Figure 1). These results reveal that the females are hemizygous and therefore manifest the phenotype, while the males are heterozygous carriers and do not, further confirming that *rdd* is in fact a sex-linked recessive trait and is not autosomal recessive as described previously [23].

Pathology: Six eyes from 6 affected birds (2 each aged 3-4 months, 9-14 months and 4 years) were examined microscopically and compared with normal controls. All eyes showed similar pathological changes, with loss and attenuation of retinal pigment epithelium, virtually complete loss of the outer nuclear layer and photoreceptors, thinning, disorganisation and gliosis of the inner nuclear layer and attenuation of the ganglion cell layer (Figure 2A,B). Additionally, there were clefts between the inner nuclear layer and retinal pigment epithelium (Figure 2C). The choroid was unaffected. In the age range examined, the severity of the condi-

Table 1. Mapping of markers to the chicken Z chromosome

Marker	Recombination fraction	Informative meioses	Genetic distance (cM)	Consensus position (cM)
MCW0055		136		20
	0.04		4.4	
ROS0072		176		38
	0.16		16.5	
rdd		178		53
	0.22		23.9	
MCW0154		150		95
	0.02		1.6	
ADL0250		148		95
	0.16		17.0	
LEI0111		168		120
	0.16		17.0	
MCW0128		160		178

Table of Z chromosome markers used in the fine mapping of the *rdd* trait. Marker order and the most likely placing for *rdd* are given in the left hand column. Subsequent columns show the recombination fractions between markers, number of meioses informative for each marker, and genetic distance between markers (calculated using the Kosambi mapping function). These distances are based on data from the crosses described here. For comparative purposes the last column shows the consensus distance from the top of the Z chromosome map for each marker as reported by Groenen et al. [21].

tion did not appear to change with age. These findings are in agreement with those of Randall and co-workers [2,3]. They observed gaps in the RPE from embryo onwards which correspond to those found here. They also observed loss of inner nuclear layer cells and severe loss of photoreceptors and outer nuclear layer cells.

Linkage analysis: Twenty-two Z chromosome markers [21] were genotyped in the *rdd* families. Details for the 6 best-linked markers are given in Table 1. Primer details can be found in the chicken database. A graph of log(relative likelihood) was plotted against genetic distance, showing the region to which *rdd* maps, to within 99% confidence limits (53±7 cM; Figure 3). The most likely position of *rdd* relative to adjacent markers is ROS0072-MCW0055-*rdd*-ADL0250-MCW0154-LEI0111-MCW0128 (Table 2). ROS0072 and MCW0154 map very close to MCW0055 and ADL0250 respectively, and are therefore not shown on the graph.

DISCUSSION

The rdd *phenotype:* Previous reports on the *rdd* trait in the early 1980's [2,3] consisted of behavioural and histopathological testing. These data led to the suggestion that this chicken disease was similar to human recessive retinitis pigmentosa. However the absence of any clinical or electrophysiological data in these papers left significant gaps in the available information on this trait. We now report a clinical description that further substantiates the link with human RP. *rdd* homozygous males (*rdd*/*rdd*) and hemizygous females (*rdd*/-) have a sluggish pupillary response, an atrophic pecten and widespread pigmentary disturbance. The defect is early in onset and severe, with affected birds having only limited vision even at hatch and a flat ERG at 3 weeks. Nevertheless there is evidence of progression in that pigmentation is more prominent and posterior subcapsular lens opacities are more common in older birds. Atrophy of the pecten, which functions as the vascular supply of the avian retina, parallels the attenuation of retinal vessels seen in human RP patients, while the atrophy frequently noted around the base of the pecten, which emerges from the optic nerve head, could equate to optic disc pallor.

Various measurements of gross eye morphology showed no significant changes from wild-type, distinguishing the *rdd* phenotype from two other chicken blindness models [12,13] in which globe enlargement is a significant feature. Pathological examination confirmed previous findings, revealing a severe retinal dystrophy involving thinning of the RPE and of all layers of the retina, and near complete loss of the outer nuclear layer and photoreceptors. However the crosses de-

TABLE 2. RELATIONSHIP OF THE RDD LOCUS TO MARKERS ON THE Z CHROMOSOME

Marker	Recombination fraction	LOD
MCW0055 - rdd	0.21	24.12
ROS0072 - rdd	0.16	33.90
MCW0154 - rdd	0.20	26.54
ADL0250 - rdd	0.24	24.92
LEI0111 - rdd	0.41	24.21
MCW0128 - rdd	0.39	20.13

Recombination values and two-point LOD scores obtained when the position of the *rdd* locus was compared against various Z chromosome markers.

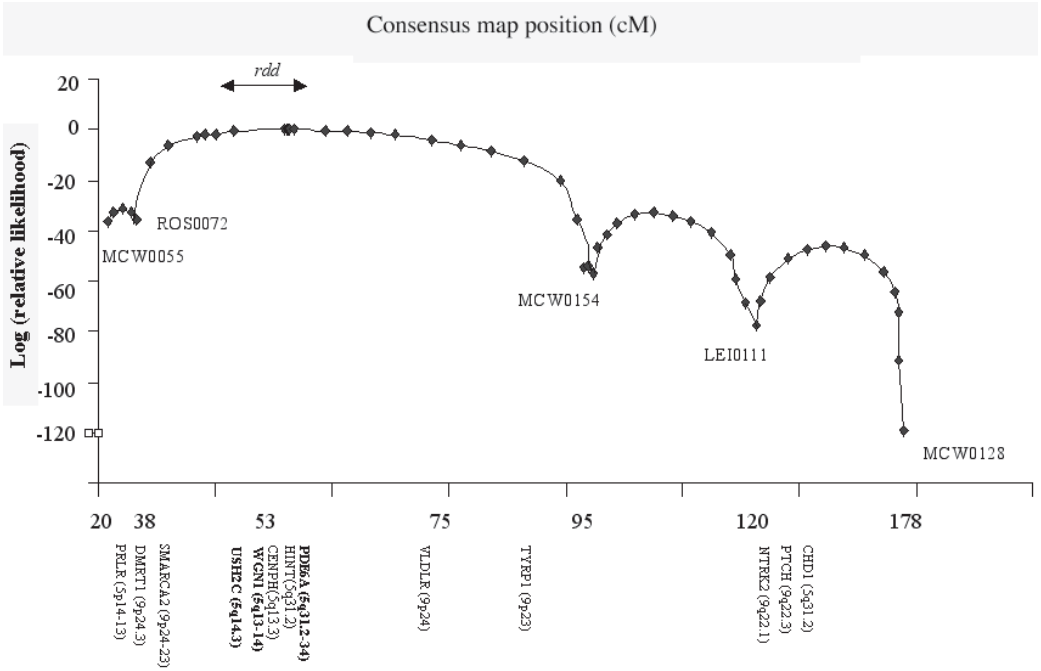

Figure 3. Mulitpoint genetic mapping of the *rdd* locus on the chicken Z chromosome. Graph of the relative probability for the location of the *rdd* locus in relation to Z chromosome markers, calculated using the Crimap program. Genes shown in normal font below the x-axis are the chicken homologues of known human genes placed on the comparative map of the chicken versus human genomes. Genes and loci shown in bold represent probable placements based on proximity to genes placed on the comparative map.

scribed herein have proved that mode of inheritance was incorrectly described in previous studies. Crosses carried out by Randall and co-workers [2], while said to confirm recessive inheritance, were in fact equally consistent with sex linkage, and we have shown that *rdd* is a Z-linked trait.

Candidate Genes: Data presented herein map the *rdd* locus to the chicken Z sex chromosome, in a region for which the best supported human homology is with chromosomes 9p24-23 and 5q13-21. There are no known retinal diseases mapping to human chromosome 9p, but two loci for human inherited retinopathies have been mapped to chromosome 5q13-21. Mutations at the *WGN1* locus mapping to 5q13-14 cause hyaloideoretinal degeneration of Wagner (Wagner syndrome type 1), a dominant condition involving abnormal vitreous structure, retinal detachment and cataracts [24,25]. The *USH2C* locus for Usher syndrome type 2, (deafness and retinitis pigmentosa in the absence of vestibular dysfunction), maps to 5q14-21 [26]. The genes underlying these disorders remain to be identified. However the recessive inheritance and pigmentary retinopathy phenotype of *rdd* argue against a link with Wagner syndrome, and the lack of any hearing deficiency makes *USH2C* a less likely candidate locus.

There is second area of synteny with the chicken Z chromosome more distally on human chromosome 5q31. This lies within the *rdd*-linked region but is outside the best-supported interval. The gene encoding the alpha sub-unit of cGMP phosphodiesterase, *PDE6A* may be predicted to lie within this region [27,28]. However, its placement is based on comparative mapping of nearby genes and remains to be confirmed. Mutations in this gene are known to cause autosomal recessive retinitis pigmentosa in humans [29,30]. *PDE6A* is one of 3 subunits of rod phosphodiesterase (PDE). In the phototransduction cascade, PDE is activated by transducin and hydrolyses cGMP to 5'GMP, causing a decrease in intracellular cGMP, which in turn causes the cGMP-gated cation channels in the outer segment membrane to close. Mutations in both the alpha and beta-subunits of rod PDE can cause retinitis pigmentosa. However further studies will be required to determine whether chicken *PDE6A* can be excluded from the *rdd* locus by genetic analysis.

In summary, the chicken *rdd* trait is a sex-linked recessive form of inherited retinal degeneration with many similarities to severe early-onset retinitis pigmentosa in humans. Linkage mapping places *rdd* in a region homologous to human chromosomes 9p and 5q. The strongest candidate gene in these regions as judged by phenotype is *PDE6A*, mutations in which cause recessive retinitis pigmentosa in humans. However, based on comparative maps with human, the chicken *PDE6A* gene is predicted to lie out with the region of highest likelihood for *rdd*. The *WGN1* and *USH2C* loci are more tightly linked but there are significant differences between these human eye diseases and chicken *rdd*. It therefore remains to be determined whether *rdd* is the direct chicken equivalent of a human retinal dystrophy or whether it represents a new disease entity.

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